Biocontrol Science and Technology (1996) 6, 435–447

CARFAX

The Genetic Improvement of Entomopathogenic Nematodes and Their Symbiont Bacteria: Phenotypic Targets, Genetic Limitations and an Assessment of Possible Hazards

ANN M. BURNELL AND BARBARA C. A. DOWDS

Department of Biology, St Patrick's College, Maynooth, Co. Kildare, Ireland

The methodologies of classical genetics and genetic engineering can be used for the genetic improvement of entomopathogenic nematodes (EPNs) and their symbiont bacteria. Many of the complex behavioural and physiological traits which are targets for genetic improvement are likely to be controlled polygenically, thus selective breeding for improvements to these traits would be appropriate. Much basic research needs to be carried out before researchers will be able to effect improvements to EPNs and their symbionts by genetic engineering. There is a lack of basic information on the genetics and biochemistry of the characteristics that might be altered by transgenic methods in EPNs, and their bacteria, and existing transformation protocols need to be made more effective.

Keywords: *selective breeding, mutagenesis, genetic engineering, pathogenicity, anhydrobiosis, host specificity, phase variation, biological containment*

INTRODUCTION

The perceived advantages of biopesticides as compared with conventional chemical pesticides have been well documented (see reviews in Gaugler & Kaya, 1990 and in Bedding *et al.*, 1993). Biopesticide products based on entomopathogenic nematodes (EPNs) and their symbiont bacteria are at present aimed almost exclusively at relatively small niche markets (e.g. protected horticulture). To increase the EPN market share, it will be necessary to develop strains of nematodes and bacteria suitable for the biological control of insect pests in non-protected horticulture and in agriculture. Strains with improvements to their pathogenicity, host range, environmental tolerances and shelf-life would be particularly desirable.

During the past 15 years, much baseline research has been carried out on the biology of EPNs and their symbiont bacteria. Many new isolates of *Steinernema* and *Heterorhabditis* have been collected worldwide and tested for their pathogenicity to various target insect pests and for their resistance to environmental extremes. Progress has been made in understanding the life cycle, taxonomy, molecular systematics and cytogenetics of these nematodes. Progress has also been made in the taxonomy and molecular systematics of the bacterial symbionts, and a greater understanding of the following processes has been obtained: bacterial phase variation, nematode–bacterial specificity and the interactions between the bacteria and the immune system of the insect host. Methods for the *in vitro* production of EPNs and their symbionts on semi-solid media

0958–3157/96/030435–13 $7.50

and in fermenters and for the formulation, storage and field application of the nematode's infective stage juvenile (IJ) have also been developed.

The increased knowledge of the basic biology of EPNs and their symbionts has led to an awareness among researchers of the potential for the genetic improvement of these organisms. The genetic studies carried out to date on *Heterorhabditis* and *Steinernema* have used conventional techniques, such as selective breeding, inbred lines and mutagenesis. Molecular approaches have been used more extensively in genetic studies of the symbiont bacteria *Xenorhabdus* and *Photorhadbus*. Several genes from these genera have been cloned and characterized, and DNA has been transferred (at low levels of efficiency) into *Xenorhabdus* and *Photorbabdus* by means of transformation, conjugation and transduction using *Escherichia coli* plasmids, mobilizable plasmids and phage λ respectively (reviewed by Dowds, 1994).

METHODOLOGY FOR GENETIC IMPROVEMENT

Classical Genetics

Most beneficial traits in farm animals and plants are inherited polygenically and the efficacy of selective breeding in the genetic improvement of such traits has been well documented (see Falconer, 1989; Willis, 1991). Glazer *et al.* (1991) found that for *H. bacteriophora* the heritability values for the traits of host-finding, ultraviolet (UV) tolerance and heat tolerance were high, indicating that artificial selection would be an appropriate method for genetic improvement of these traits in commercial strains of *Heterorhabditis*. Selective breeding programmes have been successfully applied to *Steinernema* for the traits of host-finding (Gaugler *et al.*, 1989a; Gaugler & Campbell, 1991) and improved pest control efficacy (Tomalak, 1994). Griffin and Downes (1994) reported the successful selection of *Heterorhabditis* for improved infectivity at low temperatures and Grewal and Gaugler (1995) have used artificial selection to extend the upper and lower thermal limits for infection and establishment in *H. bacteriophora* and *S. anomali*. Successful laboratory selection does not necessarily lead to improved field performance in the selected strains (Gaugler *et al.*, 1994); thus, due care must be given to the choice of the beneficial characteristic for selection and to the design of the most appropriate bioassay with which to measure the characteristic under selection. The reviews by Hastings (1994) and Westerman (1994) give further discussion of bioassay design and artificial selection of EPNs. The short generation time of EPNs facilitates selective breeding such that a selective breeding programme can, in many cases, be completed within 2 years. Thus, selective breeding is likely to remain an important component of EPN genetic improvement programmes in the future. There are no published reports on the use of artificial selection to develop improved strains of *Xenorhabdus* or *Photorhabdus*. D. J. Clarke and B. Dowds (unpublished) were, however, unsuccessful in their efforts to improve the low-temperature fitness of *Photorhabdus* by prolonged selection at 9°C.

Genotypic changes can be introduced into organisms by mutagenesis, but mutagenesis suffers from the drawback that only one or a small number of genes can be altered at any one time. An additional problem is that mutagenesis usually has the effect of inactivating genes, so it is not a very useful approach for introducing new genetic information. Mutagenesis is useful, however, for inactivating negative regulators of gene expression and can thus be a means for obtaining the constitutive expression of repressed genes. Constitutive expression of certain stress-response genes may be a means of protecting EPNs or their symbionts from environmental stresses. A further problem associated with the use of mutagenesis is that it can be difficult to design an effective selection or screening method for the characteristic in question. The design of mutant selection screens is more difficult in the diploid EPN than in their haploid symbionts. In diploid organisms the majority of mutations are recessive, and thus appropriate genetic crosses have to be carried out in order to detect recessive phenotypes.

Genetic Engineering

The impact of genetic engineering on strain improvement is most clearly seen in genetically engineered microorganisms (GEMS). GEMS were initially used as research tools, but are now

widely used in the biotechnology industry for the production of a variety of enzymes and pharmaceutical products, such as hormones, growth factors and antibiotics (see reviews in Pühler, 1993). In the past 10–15 years, remarkable progress has also been made in the expression of heterologous genes in transgenic animals, such as nematodes (Stinchcomb *et al.*, 1985; Fire, 1986), *Drosophila* (Rubin & Spradling, 1982), fish (Zhu *et al.*, 1985; reviewed by Chen & Powers, 1990), laboratory mice (Jaenish & Mintz, 1974; Palmiter & Brinster, 1985) and farm animals (reviewed by Pursel *et al.*, 1989). Spectacular progress has also been made in the genetic engineering of plants (see reviews by Gasser & Fraley, 1989 and Bartels & Nelson, 1994). The application of gene transfer techniques in animal and plant research has provided new insights into several areas of genetics and biology, such as gene structure and expression, developmental biology, disease resistance, oncology, immunology and medical genetics. Gene transfer techniques have many potential applications in plant and animal production in the areas of disease resistance, resistance to environmental stresses, improvements in growth performance and food content and in 'gene farming' (i.e. the mammary-gland-specific expression of heterologous proteins).

EPNs and their symbiont bacteria both have taxonomically related genera which are used as model organisms in molecular genetics (*Caenorhabditis elegans* and *E. coli* respectively). Thus, it has frequently been suggested that the techniques which have been developed for these model organisms could be readily adapted for use with EPNs and their symbiont bacteria, and that genetic improvement of EPNs and/or their symbionts by transgenic means will be achieved in the near future. We believe, however, that the rate of progress is likely to be relatively slow as there are many inherent difficulties to be resolved, the major one being the paucity of basic research information on the genetics and biochemistry of the characteristics that might be altered in EPNs and their bacteria. In many cases we do not know what genes might be usefully inserted into these organisms for the purposes of strain improvement. Many desirable phenotypes are likely to result from the concerted action of an array of genes and will, therefore, be less amenable to genetic engineering. Thus, it will be necessary to identify single genes whose products directly confer an advantageous phenotype to the transgenic nematode or bacterium or to target genes coding for key regulatory enzymes in critical metabolic pathways.

Protocols have been developed for the successful transformation of *C. elegans* by micro-injection of DNA into the hermaphrodite gonad (Stinchcomb *et al.*, 1985; Fire, 1986; Hope, 1994). In *C. elegans*, the introduced DNA assembles by homologous recombination into large concatemers containing hundreds of copies of the introduced gene, and these concatemers usually replicate as an extrachromosomal array. The DNA in extrachromosomal arrays is not transmitted reliably to daughter cells at each division, leading to genetic mosaicism and non-Mendelian patterns of inheritance. In order to integrate the transforming DNA into a *C. elegans* chromosome, transformants with extrachromosomal arrays are subjected to γ-irradiation and their progeny are screened for the stable transmission of the transgene (see Sugimoto *et al.*, 1994, for example). Retroviral vectors by which the transforming DNA can be stably introduced into the host genome in low copy numbers have not been developed for *C. elegans*. Exogenous genes have been found to be expressed and regulated properly from extrachromosomal arrays in *C. elegans*. (Way & Chalfie, 1988; Spence *et al.*, 1990), and this approach is now routinely used in molecular cloning (by phenotypic rescue) and in studies of the developmental expression (by means of reporter constructs) of *C. elegans* genes. Some *C. elegans* gene constructs, however, appear to function properly only when integrated at low copy number into one of the chromosomes (Fire, 1986; Fire & Waterston, 1989). The successful transformation by micro-injection of the HP88 strain of *Heterorhabditis* has been reported by Hashmi *et al.* (1995). Transcription of the reporter gene *lac-Z* driven by a *C. elegans* heat shock promoter was detected by these authors in the body and pharyngeal musculature and the hypodermis of the transformed *Heterorhabditis*.

Micro-injection protocols are technically demanding and time consuming. Thus, the development of protocols suitable for the mass transformation of *Heterorhabditis* (e.g. electroporation or micro-projectile bombardment) would be very desirable. The development of a biolistic transformation protocol for *Heterorhabditis* would have many advantages: large numbers of animals

could be treated in a single working day; the delivery of DNA directly into nuclei may increase the frequency of transformation; micro-particle bombardment can be carried out using eggs, larvae or adult nematodes; and since very little pre- or post-bombardment manipulation of the nematodes is required, nematode viability following bombardment is likely to be high.

Some progress has been made with the methodology for the transfer of DNA into *Xenorhabdus* and *Photorhabdus* (reviewed by Dowds, 1994). Some *E. coli* vectors, including several plasmids, mobilizable plasmids and phage λ, have been introduced into some strains but not others. For example, phage λ was used to transduce *X. bovienii* (Francis *et al.*, 1993) but would not transduce *Photorhadbus* sp. despite the fact that the latter species adsorbed the phage (U. Sommer & B. C. A. Dowds, unpublished). In both cases, the cells had to be engineered with the gene coding for the *E. coli* receptor protein before adsorption took place. Xu *et al.* (1991) have transformed *X. nematophilus* with plasmid pHX1, but U. Sommer and B. C. A. Dowds (unpublished) have failed to transform *Photorhabdus* spp. with this plasmid, despite being able to electroporate these strains with pBR322. Furthermore, the $CaCl_2$ transformation procedure, but not electroporation, works for *X. nematophilus* (Xu *et al.*, 1989), whereas the reverse is true for *Photorhabdus* (U. Sommer, K. O'Neill & B. C. A. Dowds, unpublished). These examples indicate that transformation methodology and cloning vectors that can be used with one strain are ineffective with others. Finally, the efficiency of the uptake of DNA by *Photorhabdus* and *Xenorhabdus* is generally poor, and renders difficult the generation of useful research tools such as transposon mutant banks. Notable exceptions are the transposon mutant banks in *X. nematophilus* (Xu *et al.*, 1991) and *X. bovienii* (Francis *et al.*, 1993).

TARGETS FOR GENETIC IMPROVEMENT

EPNs

The main targets for genetic improvement in *Heterorhabditis* and *Steinernema* are increased efficacy, resistance to environmental extremes, the development of anhydrobiotic strains and increased suitability of *Heterorhabditis* for culture in liquid fermenters.

Efficacy. EPNs can be highly pathogenic in laboratory tests, with LD_{50} values as low as 3–30 IJs, depending on the strain of nematode and the target insect (Bedding *et al.*, 1983); however, such high levels of pathogenicity are not observed in field studies. Thus, in order to provide effective control in field applications, EPNs are applied inundatively and recommended application rates are in the order of 2.5×10^9 nematodes ha^{-1}. If the efficacy of EPNs could be improved such that effective pest control could be achieved using lower numbers of IJs, this would significantly reduce the cost of EPN-based biopesticides for the end-user and would help in the development of new markets for EPN products. Efficacy can be broken down into many components, such as dispersal activity in the soil, host-finding ability, host specificity, host penetration, avoidance or suppression of the immune response of the host, successful packaging and transmission of the symbiont bacterium. Such complex behavioural and physiological traits are likely to result from the action of many interacting genes. Thus, selective breeding would be an appropriate means by which genetic improvements could be made to phenotypes considered to be important in EPN efficacy. Selection experiments are most likely to succeed if a single trait is selected or if two traits, at most, are co-selected. Several laboratory studies have shown that, under apparently favourable conditions for infection, only a proportion (in some cases as low as 13%) of individual IJs in a population are infective at any one time (Gaugler *et al.*, 1989b; Fan & Hominick, 1991; Curran & Heng, 1992). Although culture conditions can significantly influence infectivity, the proportion of IJs in a population which is infective at any one time under defined conditions appears to be species and strain related (Curran, 1993). This suggests that the control of the proportion of IJs in the population which are actively infective has a genetic basis and may be amenable to modification by genetic means. If activation into an

infective state depends on the perception of environmental cues by the IJ, then mutagenesis would seem to be the most appropriate means of modifying such a sensory activation system.

Resistance to environmental extremes. Improvements to EPN physiology and biochemistry which would increase the survival time or the infective capacity of IJs in unfavourable environments (e.g. at low or high temperatures, in conditions of low soil moisture or on foliar surfaces) would provide new opportunities for the use of EPNs in biological control. Many of the complex physiological traits which are responsible for the physiological tolerances of animals are polygenic in nature; thus, the most effective means of genetically improving such traits is by selective breeding. However, in situations where a key regulatory gene may be involved in controlling the expression of several genes in a coordinated pathway, mutagenesis may be appropriate. Griffin and Downes (1994) have reported the successful selection of *Heterorhabditis* for improved infectivity at low temperatures and O'Leary and Burnell (1995) have isolated desiccation-resistant strains of *Heterorhabditis* by mutagenesis. In cases where biochemical or molecular genetic studies clearly indicate that the product of a known gene can affect the environmental physiology of EPNs, then the construction of transgenic nematodes may yield improved strains. For example, the chilling tolerance of tobacco plants has been successfully modified by altering the degree of fatty acid unsaturation of phosphatidylglycerol in plants transformed with the gene coding for glycerol-3-acyltransferase (Murata *et al.*, 1992). EPNs are sensitive to inactivation by UV radiation (Gaugler *et al.*, 1992) and this, in combination with their poor desiccation tolerance, limits their usefulness for the biological control of insect pests on foliar surfaces. The mechanism of UV tolerance and damage repair in prokaryotes and eukaryotes is well understood at the molecular level (see reviews by Cadet *et al.*, 1992 and Strid *et al.*, 1994), so this trait may be amenable to genetic manipulation by transgenic means in EPNs.

Anhydrobiotic strains. The development of anhydrobiotic strains of *Heterorhabditis* and *Steinernema* which could be stored indefinitely in a desiccated state would be of benefit in the storage, shelf-life and distribution of EPNs. Anhydrobiosis is a widespread phenomenon. Many organisms, when subjected to environmental desiccation, undergo structural and biochemical changes which enable them to survive during the period of desiccation in a metabolically repressed state. Crowe *et al.* (1992) suggest that the synthesis of trehalose (or in higher plants, sucrose) is critical to achieving the anhydrobiotic state. Popiel and Vasquez (1991) have shown that *S. carpocapsae* synthesizes trehalose in response to osmotic desiccation; however EPNs, including *S. carpocapsae* have only a limited capacity to enter into anhydrobiosis (Womersley 1990, 1993). The work of Higa and Womersley (1993), however, demonstrates that, for the anhydrobiotic nematode *Aphelenchus avenae*, the accumulation of trehalose is not sufficient to ensure anhydrobiotic survival. These authors have found that, following the induction of trehalose accumulation upon desiccation in *A. avenae*, a further period of slow drying is required before these organisms can become fully anhydrobiotic. This additional period of slow desiccation is presumably required to allow the implementation of other adaptations which are required for anhydrobiosis. No studies have been carried out to date on the molecular genetics of anhydrobiois in animals. The molecular genetics of anhydrobiosis are under investigation in the resurrection plant *Craterostigma plantagineum* (Bartels *et al.*, 1990; Nelson *et al.*, 1994). These studies have identified a set of copy DNAs (cDNAs) belonging to 10 distinct hybridization groups that are abundantly expressed upon the onset of desiccation in *C. plantagineum*. If the genetic complexity of anhydrobiosis in animals is found to be similar to that of *C. plantagineum*, then selective breeding or mutagenesis would seem to be the most promising methods for the genetic improvement of anhydrobiotic potential in EPNs.

Increased suitability of Heterorhabditis *for fermenter culture.* Second-generation cultures of *Heterorhabditis* consist of a mixture of hermaphrodite females and of amphimictic females and males. The males and the amphimictic females are unable to copulate in the constantly agitated liquid medium of the fermenters, and are thus a loss to production (Strauch *et al.*, 1994). Thus,

the development of exclusively hermaphrodite strains of *Heterorhabditis* would greatly increase production yields. It is possible that the control of hermaphrodite/amphimictic female switching may result from the action of a single gene or small number of genes and thus be amenable to modification via mutagenesis.

Entomopathogenic Bacteria

The main targets for genetic improvement of *Xenorhabdus* and *Photorhabus* are pathogenicity, host specificity, symbiont specificity, resistance to environmental extremes such as cold or desiccation and control of phase variation.

Pathogenicity. Most pathogenicity work performed with EPNs has used the wax moth, *Galleria mellonella* as the target insect. These insects are extremely sensitive to EPNs; for example, when *G. mellonella* larvae are injected with *Xenorhabdus* or *Photorhabdus* spp. the LD_{50} is less than 3 (e.g. Akhurst, 1980; Griffin *et al.*, 1989; Jackson *et al.*, 1995). However, this does not reflect the situation with other insect species. For example, the LD_{50} for *X. bovienii* is 40 000 for *Tipula oleracea* (Wulff *et al.*, 1993) and *X. nematophilus* was relatively non-pathogenic to *Popillia japonica* (Yeh & Alm, 1992).

There are two main classes of insect-pathogenic bacteria: spore formers and non-spore formers. *Xenorhabdus* and *Photorhabdus* fit into the class of non-spore formers. This group is usually pathogenic as a result of the production of extracellular enzymes and/or lipopolysaccharides (LPS) that destroy the haemocytes and internal organs of the insect after the bacteria have reached the haemocoel (Lysenko, 1985). Little work has been done on characterizing the virulence determinants of *Xenorhabdus* and *Photorhabdus*. They both secrete lipases, lecithinases and proteases, and Clarke and Dowds (1995) have shown that the secreted lipase contributes to the virulence of *Photorhabdus* sp. towards *G. mellonella*. Lipase did not fully account for the toxicity of spent medium, so it is clear that other toxic molecules are secreted. The cell membrane fraction of *Xenorhabdus* was also toxic when injected into larvae, but no more so than membrane fractions from *E. coli* or *Bacillus subtilis*, whose viable cells are non-virulent towards these insects. It was proposed that this non-specific toxicity may derive from activation of the insect immune response leading to damage of its own tissues. LPS is released from living and dead bacteria, but only when they are exposed to larval serum. LPS causes haemocyte damage and inhibits the activation of prophenoloxidase, part of the insect humoral immune response (Dunphy & Webster, 1988a,b, 1991). However, when injected into *G. mellonella* LPS failed to kill any of the larvae (Clarke & Dowds, 1991). Dunphy (1994, 1995) has generated virulence mutants of *Xenorhabdus* and *Photorhabdus*, and found that virulence reflects tolerance to the host's antibacterial defences. In summary, virulence might be improved by increased toxin activity or specificity or improved avoidance by cells of various components of the insect immune response. However, much basic research is needed to elucidate the mechanisms of virulence.

Host specificity. It is clear from the different LD_{50} values mentioned above that *Xenorhabdus* and *Photorhabdus* have different degrees of virulence for different insect species. Further work needs to be carried out to assess their virulence towards a wide range of both insect pests and beneficial insects. In addition, the biochemical basis of this difference in the sensitivity of different insect species has not been investigated. In the absence of this information, there is no possibility of genetically engineering bacteria for specificity for certain insect pests while ensuring that beneficial insects are unharmed. One possible type of structure of importance for bacterial adhesion to host tissue is the fimbriae on the bacterial surface (Brehelin *et al.*, 1993). In other bacterial species, fimbriae are known to play a major role in host specificity because of their role in adhesion to surfaces.

Symbiont specificity. Studies have shown that it is possible, in some cases, to grow *Steinernema* and *Heterorhabditis* on bacteria other than their own specific symbionts (Akhurst, 1983; Dunphy

et al., 1985; Ehlers *et al.*, 1990; Han *et al.*, 1990, 1991; Gerritsen & Smits, 1993). When symbionts were exchanged between *Heterorhabditis* species and strains, only some nematode bacterial combinations were compatible (Han *et al.*, 1990; Gerritsen & Smits, 1993) and in some cases bacterial strains which could support nematode growth were not packaged by the nematodes, resulting in non-pathogenic IJs. Han *et al.* (1991) have shown that by exchanging bacteria between compatible *Heterorhabditis* strains, the pathogenicity of these strains was increased or decreased, depending on the bacteria they were grown on. Thus, although there is a degree of specificity for the bacterial strain which will support growth of, and be carried by, a given nematode strain, some scope exists for the improvement of EPN pathogenicity by making different combinations of bacteria and nematodes.

Resistance to extremes in the environment. EPNs are ineffective at the low temperatures prevailing in temperate climates in spring and autumn. Work on the adaptation of *Photorhabdus* and *X. nematophilus* to low temperatures (Clarke & Dowds, 1994a; Leisman *et al.*, 1995) and a review of the differences between psychrophiles and mesophiles and of low temperature shock in other species of bacteria (Clarke & Dowds, 1994b) suggests that many genes are involved in low temperature adaptation. These might include genes coding for cold shock and outer membrane proteins and are expected to include enzymes involved in desaturation of membrane fatty acids. By analogy to other stress responses, it is possible that a small number of regulatory genes are involved in controlling expression of the adaptive genes, suggesting that mutations in the regulatory genes might yield cold-active mutant strains. However, it is not known whether such strains would display low temperature virulence as well as low temperature growth.

Another stress adaptation that might be usefully improved upon is the response to desiccation. It is known that the nematode is sensitive to drying, but the sensitivity of the bacteria has not yet been determined. A range of adaptations of bacteria to desiccation have been demonstrated or hypothesized, including trehalose production, membrane structure and composition and capsule formation (Potts, 1994). Nothing is known about these characteristics in *Xenorhabdus* and *Photorhabdus* except that these bacteria have fluid membranes typical of psychrotrophs (Clarke & Dowds, 1994b; Janse & Smits, 1990; Suzuki *et al.*, 1990) and that the primary forms synthesize capsules (Brehelin *et al.*, 1993).

Phase variation. *Xenorhabdus* and *Photorhabdus* display a form of phenotypic instability known as phase variation. Thus, a culture grown from a single colony of primary phase (phase I) generates secondary forms (phase II) at a rate that is highly dependent on the strain. This has proved to be a major nuisance in the industrial production of the nematodes because the yield of nematodes from growth on secondary-form bacteria is only a fraction (one seventh in the reference cited) of that from primary forms (Akhurst, 1980). In addition, the primary form is preferentially retained by the infective stage nematode. The reason for these differences may be that the secondary form fails to produce a range of enzymes and secondary metabolites made by the primary form, including extracellular protease, lipase, antibiotics, fimbriae, capsule and many other products. Possibly because of the conservation of energy by the secondary form, it can recommence growth following starvation much more rapidly than the primary form, indicating a more efficient nutrient uptake system. The secondary form also has higher levels of respiratory enzymes (Smigielski *et al.*, 1994). These authors suggest that the secondary phase may be better adapted to conditions in soil as free-living bacteria. Other recent work suggests another alternative. Krasomil-Osterfeld and Ehlers (1994) have shown that a decrease in medium osmolarity can induce at least some of the characteristics of the secondary phase in primary-phase cultures. They showed that the osmolarity of phase II-inducing medium was the same as that within the nematode gut, whereas the osmolarity of phase I-inducing medium was the same as that in the insect haemocoel. This work suggests that many bacterial characteristics are switched off in the nematode, possibly because they would be detrimental, e.g. digest the host tissue. While the primary-phase-specific characteristics seem likely to be involved in virulence (this has been demonstrated in the case of lipase), the relationship is not simple. Secondary forms

of *X. nematophilus* have been shown to be equally virulent to the primaries after injection into *G. mellonella* larvae (Akhurst, 1980). However, Jackson *et al.* (1995) found that the primary phase of *Photorhabdus* sp. was about six times more virulent than the secondary, but each had an LD_{50} value of less than 1 for *G. mellonella* larvae at 28°C. It would be desirable to use a less sensitive host in order to resolve this point.

It seems apparent that mutating *Xenorhabdus* and *Photorhabdus* into a stable primary form would be advantageous to the industrial production of these bacteria. However, the role of the secondary form in the life cycle or in adapting to changes in the environment needs to be elucidated before we can be certain that such mutants would be advantageous when applied in the field. Many pathogens, and possibly most bacterial populations, undergo various forms of phase variation, all of which assist the bacteria in adapting to the variety of conditions that they encounter (Robertson & Meyer, 1992; Brunham *et al.*, 1993). It is unlikely that such a complex change in metabolism is without major adaptive implications for *Xenorhabdus* and *Photorhabdus* as well. There may also be problems with the technology of generating a mutant that is completely locked into the primary phase. Work is accumulating which suggests that there are multiple regulatory controls affecting the phase shift. There may be a single regulatory gene controlling a number of other regulators, but so far there is no evidence for this. The inactivation of phase variant characteristics seems to be regulated at a post-translational level for lipase and protease (Wang & Dowds, 1993) but might be regulated by antisense RNA in the case of bioluminescence as suggested by Hosseini (1994). An intermediate form (J. Zeller & B. C. A. Dowds, unpublished), a mutant (Hosseini, 1994), low-salt media (Krasomil-Osterfeld & Ehlers, 1994; D. McDonagh & B. C. A. Dowds, unpublished) and rifampicin (Hosseini, 1994; D. Roche & B. C. A. Dowds, unpublished) all affect some, but not all, phase variant characteristics (different sets in each case). Thus, any postulated primary level of control of the phase variant phenotypes must have a variable effect on the different characteristics, if indeed such an overall control exists.

RISKS OF RELEASE OF GENETICALLY MODIFIED EPNS AND/OR THEIR SYMBIONT BACTERIA

While the field release of genetically modified EPNs and bacteria may be beneficial in terms of improved pest control, access to new target pests and a reduction in the use of less selective and more toxic pesticides, the release of such genetically engineered organisms (GEOs) will pose some risks which need to be assessed by regulatory authorities. The ecological risks posed by the introduction into the environment of GEOs have been discussed in several recent reviews (Tiedje *et al.*, 1989; Fincham & Ravetz, 1991; Gustafsson & Jansson, 1993; Molin & Kjellenberg, 1993; Wilson & Lindow, 1993; Regal, 1994). The possible risks are:

(1) the transfer of foreign genes to other organisms in the ecosystem, especially to deleterious organisms such as pathogens and pests; and
(2) the possibility that the GEOs will survive better than the parent strain in some environments, with unknown consequences.

The occurrence of horizontal gene transfer in bacteria by means of conjugation, transformation and transduction has been well documented (see review by Syvanen, 1994). Natural transformation and phage-mediated transduction usually mediate narrow host-range transfer between strains of the same bacterial species, whereas broad host-range DNA transfer in microorganisms is usually mediated by conjugation, and there are now several examples of inter-genus and even inter-kingdom gene transfer via conjugation (reviewed by Salyers & Shoemaker, 1994). There is strong evidence to support the horizontal transfer of P-factor transposons in insects (Clark *et al.*, 1994) and it is likely that other families of transposable elements may also have undergone horizontal transfer during phylogeny; however, the available evidence suggests that horizontal transfer of DNA to animals is extremely rare in nature. Of nine putative examples of horizontal nuclear gene transfer involving eukaryotes cited by Syvanen (1994), an animal host (the protzoan

Entamoeba histolytica) was only involved in one case. Thus, the potential risks of horizontal gene transfer from GEOs to other organisms in the ecosystem seem to be less for genetically engineered nematodes than for genetically engineered bacteria.

There are no reports of the isolation of *Xenorhabdus* and *Photorhabdus* from soil samples, so it seems likely that bacteria may not be released from the nematodes or insects into the soil in significant numbers and/or that the bacteria may not survive for significant lengths of time in the free state. No systematic studies have, however, been carried out to investigate either the occurrence of free bacteria in the soil or the survival capacity of *Xenorhabdus* and *Photorhadbus* in the soil. If experimental evidence can be obtained which demonstrates that wild-type *Xenorhabdus* and *Photorhabdus* do, indeed, survive poorly in the free state, such information would have a major impact on the assessment of the potential risk of horizontal transfer of transgenes from these organisms. Thus, such experiments urgently need to be carried out. If, however, it is found that wild-type *Xenorhabdus* and *Photorhabdus* can survive in the free state in the soil, then the possible risks of their release into the environment need to be considered. Such risks may be divided into problems associated with the release of non-engineered strains and risks arising from recombinant strains. Wilson and Lindow (1993) have reviewed the current applications of microorganisms in the environment and the history of the release of non-engineered microbes. These workers have made an assessment of the fate of released recombinants. Deliberate release of non-engineered microorganisms has not caused any obvious problems, but few of the studies have attempted to describe the fate of the introduced species. The authors note that currently we do not have the tools for the sensitive assay of community function and thus we cannot adequately study the effects of the release of organisms including GEMs. We know from laboratory studies that *Photorhabdus* can receive genes from *E. coli* by conjugation, and that it contains bacteriophage and plasmids which could infect or transform other species (see Dowds, 1994 for a review). A specific risk from such events might be the generation of insect-pathogenic species of bacteria from non-pathogens, with a possibly broader host range. The most obviously damaging target would be beneficial insect species, such as pollinators, but the infection of other species is not impossible. For example, a strain of *Photorhabdus* has been isolated from human wounds, where they clearly survive, though there is no evidence that they are pathogenic (Colepicolo *et al.*, 1989).

Molin *et al.* (1993) have reviewed the requirements and strategies for biological containment of released bacteria. They discuss two approaches—that of the introduction of disabling mutations into strains to be released and that of the introduction of suicide genes expressed from a controlled promoter. There are two problems in the former approach:

(1) the handicapping of a species in performing its task, e.g. in pest control;
(2) the difficulty of obtaining the appropriate mutant for a poorly studied species.

With respect to the latter approach, conditions that might be used to regulate expression of the killing gene in the soil include low temperatures, starvation or stochastic control by random recombinational switches. One problem with the suicide gene approach is that, in a laboratory situation at least, a subpopulation of the culture survives expression of the killing gene. These survivors are usually spontaneous mutants affected in either the killing gene or another gene. Thus, it is possible to design strains whose numbers can be greatly reduced (e.g. by a factor of a million) under specific conditions, but it is not possible to eliminate totally a population of released bacteria. For lower risk situations, reduction rather than elimination may constitute an adequate safety solution.

Ehlers and Peters (1995) have evaluated the possible environmental impacts and risks of the inundative release of non-engineered EPN for the control of insect pests. Their salient observations are:

(1) that *Steinernema* and *Heterorhabditis* are limited to the soil environment and their host spectrum is limited to arthropods, particularly insects;
(2) all available evidence indicates that the application of EPNs has an insignificant impact on

non-target hosts, including beneficial insects (the majority of beneficial insects do not have soil-dwelling larvae);
(3) following inundative release, EPN numbers decline rapidly;
(4) EPN population dynamics are influenced by soil-dwelling nematophagous antagonists such as fungi and invertebrate predators.

Thus, the available experimental evidence and the experience gained in the commercial exploitation of EPNs in pest control suggests that the use of non-engineered EPNs for the control of insect pests poses minimal threat to ecosysems. The question is whether genetically engineered EPNs or their symbionts would survive better than their parent strain in some environments, with unknown, but possibly deleterious, consequences to the ecosystem. Due to the technical difficulties involved in the engineering of EPNs and their symbionts, it is likely that, at least in the initial stages, transgenic EPNs and bacteria will differ from their parent strains by only a single gene or by small numbers of genes. Thus, it is likely that the release of such GMOs would pose less threat than the release of non-native EPN species, since the latter would result in the release of a greater amount of novel genes into the ecosystem. As Tiedje *et al.* (1989) point out, however, the important consideration is the character of the phenotypic changes expressed after the manipulation of the organism's genotype and not simply how many genes have been added or deleted.

ACKNOWLEDGEMENTS

Work in the authors' laboratories is supported by the European Community (STD-3 Programme contract TS3 CT94-0273, A. M. B.; European Union Human Capital and Mobility Institutional Fellowship Contract CT 930486, A. M. B. & B. C. A. D.) and by Forbairt, the Irish Science and Technology Agency (A. M. B. & B. C. A. D).

REFERENCES

AKHURST, R.J. (1980) Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* **121,** 303–309.

AKHURST, R.J. (1983) *Neoaplectana* species: specificity of association with bacteria of the genus *Xenorhabdus*. *Experimental Parasitology* **55,** 258–263.

BARTELS, D. & NELSON, D. (1994) Approaches to improve stress tolerance using molecular genetics. *Plant Cell and Environment* **17,** 659–667.

BARTELS, D., SCHNEIDER, K., TERSTAPPEN, G., PIATOWSKI, D. & SALAMINI, F. (1990) Molecular cloning of abscisic acid modulated genes which are induced during desiccation of the resurrection plant *Craterostigma plantagineum*. *Planta* **181,** 27–34.

BEDDING, R.A, MOLYNEUX, A.S. & AKHURST, R.J. (1983) *Heterorhabditis* spp., *Neoaplectana* spp. and *Steinernema kraussei*: interspecific differences in infectivity for insects. *Experimental Parasitology* **55,** 259–257.

BEDDING, R., AKHURST, R. & KAYA, H. (Eds) (1993) *Nematodes and the Biological Control of Insect Pests*. CSIRO Publications, East Melbourne, Australia.

BREHELIN, M., CHERQUI, A., DRIF, L., LUCIANI, J., AKHURST, R. & BOEMARE, N. (1993) Ultrastructural study of surface components of *Xenorhabdus* sp. in different cell phases and culture conditions. *Journal of Invertebrate Pathology* **61,** 188–191.

BRUNHAM, R.C., PLUMMER, F.A. & STEPHENS, R.S. (1993) Bacterial antigenic variation, host immune response, and pathogen–host coevolution. *Infection and Immunity* **61,** 2273–2276.

CADET, J., ANSELMINO, C., DOUKI, T. & VOITURIEZ, L. (1992) Photochemistry of nucleic acids in cells. *Journal of Photochemistry and Photobiology B-Biology* **15,** 277–298.

CHEN, T.T. & POWERS, D.A. (1990) Transgenic fish. *Trends in Biotechnology* **8,** 209–215.

CLARK, J.B., MADDISON, W.D. & KIDWELL, M.G. (1994) Phylogenetic analysis supports horizontal transfer of P transposable elements. *Molecular Biology and Evolution* **11,** 40–50.

CLARKE, D.J. & DOWDS, B.C.A. (1991) Pathogenicity of *Xenorhabdus luminescens*. *Biochemical Society Transactions* **20,** 65S.

CLARKE, D.J. & DOWDS, B.C.A. (1994a) The gene coding for polynucleotide phosphorylase in *Photorhabdus* sp. strain K122 is induced at low temperatures. *Journal of Bacteriology* **176,** 3775–3784.

CLARKE, D.J. & DOWDS, B.C.A. (1994b) Cold adaptation in *Photorhabdus* spp., in *Genetics of Entomopathogenic Nematode–Bacterium Complexes* (BURNELL, A.M., EHLERS, R.-U. & MASSON, J.P., Eds) European Commission Publication EUR 15681 EN, Luxembourg, pp. 170–177.

CLARKE, D.J. & DOWDS, B.C.A. (1995) Virulence mechanisms of *Photorhabdus* sp. strain K122 towards wax moth larvae. *Journal of Invertebrate Pathology*, **66,** 149–155.

COLEPICOLO, P., CHO, K.-W., POINAR, G.C., JR & HASTINGS, J. (1989) Growth and luminescence of the bacterium *Xenorhabdus luminescens* from a human wound. *Applied and Environmental Microbiology* **55,** 2601–2606.

CROWE, J.H., HOEKSTRA, F.A. & CROWE, L.M. (1992) Anhydrobiosis. *Annual Review of Physiology* **54,** 579–599.

CURRAN, J. (1993) Post application biology of entomopathogenic nematodes in soil, in *Nematodes and the Biological Control of Insect Pests* (BEDDING, R., AKHURST, R. & KAYA, H., Eds) CSIRO Publications, East Melbourne, Australia, pp. 67–77.

CURRAN, J. & HENG, J. (1992) Comparison of three methods for estimating the number of entomopathogenic nematodes present in soil samples. *Journal of Nematology* **24,** 172–176.

DOWDS, B.C.A. (1994) Molecular genetics of *Xenorhabdus* and *Photorhabdus*. *Proceedings of the Sixth International Colloquium on Invertebrate Pathology and Microbial Control, Society for Invertebrate Pathology*, Montpellier, **1,** pp. 95–100.

DUNPHY, G.B. (1994) Interaction of mutants of *Xenorhabdus nematophilus* (Enterobacteriaceae) with antibacterial systems of *Galleria mellonella* larvae (Insecta: Pyralidae). *Canadian Journal of Microbiology* **40,** 161–168.

DUNPHY, G.B. (1995) Physicochemical properties and surface components of *Photorhabdus luminescens* influencing bacterial interaction with non-self response systems of nonimmune *Galleria mellonella* larvae. *Journal of Invertebrate Pathology* **65,** 25–34.

DUNPHY, G.B. & WEBSTER, J.M. (1988a) Lipopolysaccharides of *Xenorhabdus nematophilus* (Enterobacteriaceae) and their haemocyte toxicity in non-immune *Galleria mellonella* (Insecta: Lepidoptera) larvae. *Journal of General Microbiology* **134,** 1017–1028.

DUNPHY, G.B. & WEBSTER, J.M. (1988b) Virulence mechanisms of *Heterorhabditis heliothidis* and its bacterial associate, *Xenorhabdus luminescens*, in non-immune larvae of the greater wax moth, *Galleria mellonella*. *International Journal of Parasitology* **18,** 729–737.

DUNPHY, G.B. & WEBSTER, J.M. (1991) Antihemocytic surface components of *Xenorhabdus nematophilus* var. *dutki* and their modification by serum of nonimmune larvae of *Galleria mellonella*. *Journal of Invertebrate Pathology* **58,** 40–51.

DUNPHY, G.B., RUTHERFORD, T.A. & WEBSTER, J.M. (1985) Growth and virulence of *Steinernema glaseri* influenced by different subspecies of *Xenorhabdus nematophilus*. *Journal of Nematology* **17,** 476–482.

EHLERS, R.-U. & PETERS, A. (1995) Entomopathogenic nematodes in biological control: feasibility, perspectives and possible risks, in *Biological Control: Benefits and Risks* (HOKKANEN, H.M.T. & LYNCH, J.M., Eds) Cambridge University Press, Cambridge, pp. 119–136.

EHLERS, R.-U., STOESSEL, S. & WYSS, U. (1990) The influence of phase variants of *Xenorhabdus* spp. on the propogation of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*. *Revue de Nématologie* **13,** 417–424.

FALCONER, D.S. (1989) *Introduction to Quantitative Genetics*, 3rd Edn, Longman, Harlow.

FAN, X. & HOMINICK, W.M. (1991) Efficiency of *Galleria* (wax moth) baiting technique for recovering infective stages of entomopathogenic rhabditids (Steinernematidae and Heterorhabditidae) from sand and soil. *Revue de Nématologie* **24,** 381–388.

FINCHAM, J.R.S. & RAVETZ, J.R. (1991) *Genetically Engineered Organisms: Benefits and Risks*. Open University Press, Milton Keynes.

FIRE, A. (1986) Integrative transformation of *Caenorhabditis elegans*. *The EMBO Journal* **5,** 2673–2680.

FIRE, A. & WATERSTON, R.H. (1989) Proper expression of myosin genes in transgenic nematodes. *The EMBO Journal* **8,** 3419–3428.

FRANCIS, M., PARKER, A.F., MORONA, R. & THOMAS, C.J. (1993) Bacteriophage lambda as a delivery vector for Tn10-derived transposons in *Xenorhabdus bovienii*. *Applied and Environmental Microbiology* **59,** 3050–3055.

GASSER, C.S. & FRALEY, R.T. (1989) Genetically engineering plants for crop improvement. *Science* **244,** 1293–1299.

GAUGLER, R. & CAMPBELL, J.F. (1991) Selection for enhanced host-finding of scarab larvae (Coleoptera: Scarabaeidae) in an entomopathogenic nematode. *Environmental Entomology* **20,** 700–706.

GAUGLER, R. & KAYA, H.K. (Eds) (1990) *Entomopathogenic Nematodes in Biological Control*. CRC Press, Boca Raton, FL.

GAUGLER, R., CAMPBELL, J.F. & MCGUIRE, T.R. (1989a) Genetic selection for host finding in *Steinernema feltiae*. *Journal of Invertebrate Pathology* **54,** 363–372.

GAUGLER, R., MCGUIRE, T.R. & CAMPBELL, J.F. (1989b) Genetic variability among strains of the entomopathogenic nematode *Steinernema feltiae*. *Journal of Nematology* **21,** 247–253.

GAUGLER, R., BEDNAREK, A. & CAMPBELL, J.F. (1992) Ultraviolet inactivation of heterorhabditid and steinernematid nematodes. *Journal of Invertebrate Pathology* **59,** 155–160.

GAUGLER, R., GLAZER, I., CAMPBELL J.F. & LIRAN, N. (1994) Laboratory and field evaluation of an entomopathogenic nematode genetically selected for improved host-finding. *Journal of Invertebrate Pathology* **63,** 68–73.

GERRITSEN, L.J.M. & SMITS, P.H. (1993) Variation in pathogenicity of recombinations of *Heterorhabditis* and *Xenorhabdus luminescens* strains. *Fundamental and Applied Nematology* **16,** 367–373.

GLAZER, I., GAUGLER, R. & SEGAL, D. (1991) Genetics of the nematode *Heterorhabditis bacteriophora* strain HP88: the diversity of beneficial traits. *Journal of Nematology* **23,** 324–333.

GREWAL, P. & GAUGLER, R. (1995) Thermal niches of entomopathogenic nematodes are malleable. *Proceedings of the Twenty-eighth Meeting of the Society for Invertebrate Pathology*, Cornell University, 16–21 July 1995, p. 24.

GRIFFIN, C.T. & DOWNES, M.J. (1994) Selection of *Heterorhabditis* sp. for improved infectivity at low temperatures, in *Genetics of Entomopathogenic Nematode–Bacterium Complexes* (BURNELL, A.M., EHLERS, R.-U. & MASSON, J.P. Eds) European Commission Publication EUR 15681 EN, Luxembourg, pp. 143–151.

GRIFFIN, C.T., SIMONS, W.R. & SMITS, P.H. (1989) Activity and infectivity of four isolates of *Heterorhabditis* spp. *Journal of Invertebrate Pathology* **53,** 107–112.

GUSTAFSSON, K. & JANSSON, J.K. (1993) Ecological risk assessment of the deliberate release of genetically modified microorganisms. *Ambio* **22,** 236–242.

HAN, R., WOUTS, W.M. & LI, L. (1990) Development of *Heterorhabditis* spp. strains as characteristics of possible *Xenorhabdus luminescens* subspecies. *Revue de Nématologie* **13,** 411–415.

HAN, R., WOUTS, W.M. & LI, L. (1991) Development of *Heterorhabditis* spp. strains associated with different *Xenorhabdus luminescens* isolates. *Journal of Invertebrate Pathology* **58,** 27–32.

HASHMI, S., HASHMI, G. & GAUGLER, R. (1995) Genetic transformation of an entomopathogenic nematode by microinjection. *Journal of Invertebrate Pathology* **66,** 293–296.

HASTINGS, I.M. (1994) Introduction to quantitative genetics: inbreeding, heritability estimates and artificial selection, in *Genetics of Entomopathogenic Nematode–Bacterium Complexes* (BURNELL, A.M., EHLERS, R.-U. & MASSON, J.P., Eds) European Commission Publication EUR 15681 EN Luxembourg, pp. 120–128.

HIGA, L.M. & WOMERSLEY, C.Z. (1993) New insights into the anhydrobiotic phenomenon: the effects of trehalose content and differential rates of evaporative water loss on the survival of *Aphelenchus avenae*. *The Journal of Experimental Zoology* **267,** 120–129.

HOPE, I.A. (1994) Genetic transformation of *Caenorhabditis elegans*, in *Genetics of Entomopathogenic Nematode–Bacterium Complexes* (BURNELL, A.M., EHLERS, R.-U. & MASSON, J.P., Eds) European Commission Publication EUR 15681 EN, Luxembourg, pp. 14–22.

HOSSEINI, P. (1994) Regulation of bioluminescence in *Photorhabdus* HM, primary and secondary forms. MSc thesis, University of Wisconsin, Milwaukee, WI.

JACKSON, T.J., WANG, H., NUGENT, M.J., GRIFFIN, C.T., BURNELL, A.M. & DOWDS, B.C.A. (1995) Isolation of insect pathogenic bacteria, *Providencia rettgeri*, from *Heterorhabditis* spp. *Journal of Applied Bacteriology* **78,** 237–244.

JAENISH, R. & MINTZ, B. (1974) Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. *Proceedings of the National Academy of Sciences USA* **240,** 1250–1254.

JANSE, J.D. & SMITS, P.H. (1990) Whole cell fatty acid patterns of *Xenorhabdus* species. *Letters in Applied Microbiology* **10,** 131–135.

KRASOMIL-OSTERFELD, K. & EHLERS, R.-U. (1994) Influence of environmental factors on phase variation in *Photorhabdus luminescens*. *Proceedings of the Sixth International Colloquium on Invertebrate Pathology and Microbial Control*, Society for Invertebrate Pathology, Montpellier, **1,** pp. 101–106.

LEISMAN, G.B., WAKAU, J. & FORST, S.A. (1995) Characterisation and environmental regulation of outer membrane proteins in *Xenorhabdus nematophilus*. *Applied and Environmental Microbiology* **61,** 200–204.

LYSENKO, O. (1985) Non-sporeforming bacteria pathogenic to insects: incidence and mechanisms. *Annual Review of Microbiology* **39,** 673–695.

MOLIN, S. & KJELLEBERG, S. (1993) Release of engineered microorganisms: containment and improved predictability for risk assessment. *Ambio* **22,** 242–245.

MOLIN, S., BOE, L., JENSEN, L.B., KRISTENSEN, C.S., GIVSKOV, M., RAMOS, J.L. & BEJ, A.K. (1993) Suicidal genetic elements and their use in biological containment of bacteria. *Annual Review of Microbiology* **47,** 139–166.

MURATA, N., ISHIZAKI-NISHIZAWA, O., HIGASHI, S., HAYASHI, H., TASAKA, Y. & NISHIDA, I. (1992) Genetically engineered alteration in the chilling sensitivity of plants. *Nature* **356,** 710–713.

NELSON, D., SALAMINI, F. & BARTELS, D. (1994) Abscisic acid promotes novel DNA-binding activity to a desiccation-related promoter of *Craterostigma plantagineum*. *The Plant Journal* **5,** 451–458.

O'LEARY, S.A. & BURNELL, A.M. (1995) The genetic improvement of desiccation tolerance in the insect parasitic nematode *Heterorhabditis*. *Proceedings of the Twenty-second International Symposium of The European Society of Nematologists*, Ghent, Belgium, 7–14 August, 1994. *Nematologica* **41,** 327.

PALMITER, R.D. & BRINSTER, R.L. (1985) Transgenic mice. *Cell* **41,** 237–244.

POPIEL, I. & VASQUEZ, E.M. (1991) Cryopreservation of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. *Journal of Nematology* **23,** 432–437.

POTTS, M. (1994) Desiccation tolerance of prokaryotes. *Microbiological Reviews* **58,** 755–805.

PÜHLER, A. (Ed.) (1993) *Genetic Engineering of Microorganisms*. VCH, Weinheim, Germany.

PURSEL, V.G., PINKERT, C.A., MILLER, K.F., BOLT, D.J., CAMBBELL, R.G., PALMITER, R.D., BRINSTER, R. & HAMMER, R.E. (1989) Genetic engineering of livestock. *Science* **244,** 1281–1288.

REGAL, P.J. (1994) Scientific principles for ecologically based risk assessment of transgenic organisms. *Molecular Ecology* **3,** 5–13.

ROBERTSON, B.D. & MEYER, T.F. (1992) Genetic variation in pathogenic bacteria. *Trends in Genetics* **8,** 422–427.

RUBIN, G.M. & SPRADLING, A.C. (1982) Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218,** 348–353.

SALYERS, A.A. & SHOEMAKER, N.B. (1994) Broad host range gene transfer: plasmids and conjugative transposons. *FEMS Microbiology Ecology* **15,** 15–22.

SMIGIELSKI, A.J., AKHURST, R.J. & BOEMARE, N.E. (1994) Phase variation in *Xenorhabdus nematophilus* and *Photorhabdus luminescens*: differences in respiratory activity and membrane energization. *Applied and Environmental Microbiology* **60,** 120–125.

SPENCE, A.M., COULSON, A. & HODGKIN, J. (1990) The product of *fem-1*, a nematode sex-determining gene contains a motif found in cell cycle control proteins and receptors for cell–cell interactions. *Cell* **60,** 981–990.

STINCHCOMB, D.T., SHAW, J.E., CARR, S.H. & HIRSH, D. (1985) Extrachromosomal DNA transformation of *Caenorhabditis elegans. Molecular and Cellular Biology* **5,** 3484–3496.

STRAUCH, O., STOESSEL, S. & EHLERS, R.-U. (1994) Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis. Fundamental and Applied Nematology* **17,** 575–582.

STRID, A., CHOW, W.S. & ANDERSON, J.M. (1994) UV-B damage and protection at the molecular level in plants. *Photosynthesis Research* **39,** 475–489.

SUGIMOTO, A., FRIESEN, P.D. & ROTHMAN, J.H. (1994) Baculovirus p35 prevents developmentally programmed cell death and rescues a ced-9 mutant in the nematode *Caenorhabditis elegans. The EMBO Journal* **13,** 2023–2028.

SUZUKI, T., YAMANAKA, S. & NISHIMURA, Y. (1990) Chemotactic study of *Xenorhabdus* species—cellular fatty acids, ubiquinone and DNA–DNA hybridization. *Journal of General and Applied Microbiology* **36,** 393–401.

SYVANEN, M. (1994) Horizontal gene transfer: evidence and possible consequences. *Annual Review of Genetics* **28,** 237–261.

TIEDJE, J.M., COLWELL, R.K., GROSSMAN, Y.L., HODSON, R.E., LENSKI., R.E., MACK, R.N. & REGAL, P.J. (1989) The planned introduction of genetically engineered organisms: ecological considerations and recommendations. *Ecology* **70,** 298–315.

TOMALAK, M. (1994) Selective breeding of *Steinernema feltiae* (Filipjev) (Nematoda, Steinernematidae) for improved efficacy in control of a mushroom fly *Lycoriella solani* Winnertz (Diptera, Sciaridae). *Biocontrol Science and Technology* **4,** 187–198.

WANG, H. & DOWDS, B.C.A. (1993) Phase variation in *Xenorhabdus luminescens*: cloning and sequencing of the lipase gene and analysis of its expression in primary and secondary phases of the bacterium. *Journal of Bacteriology* **175,** 1665–1673.

WAY, J.C. & CHALFIE, M. (1988) *Mec-3*, a homeobox-containing gene that specifies differentiation in touch receptor neurons in *C. elegans. Cell* **54,** 5–16.

WESTERMAN, P.R. (1994) An essay on assays, in *Genetics of Entomopathogenic Nematode–Bacterium Complexes* (BURNELL, A.M., EHLERS, R.-U. & MASSON, J.P., Eds) European Commission Publication EUF 15681 EN, Luxembourg, pp. 129–142.

WILLIS, M.B. (1991) *Dalton's Introduction to Practical Animal Breeding*, 3rd Edn. Blackwell Scientific Publications, London.

WILSON, M. & LINDOW, S.E. (1993) Release of recombinant microorganisms. *Annual Review of Microbiology* **47,** 913–944.

WOMERSLEY, C.Z. (1990) Dehydration survival and anhydrobiotic potential, in *Entomopathogenic Nematodes in Biological Control* (GAUGLER, R. & KAYA, H.K., Eds) CRC Press, Boca Raton, FL, pp. 117–137.

WOMERSLEY, C.Z. (1993) Factors affecting physiological fitness and modes of survival employed by dauer juveniles and their relationship to pathogenicity, in *Nematodes and the Biological Control of Insect Pests* (BEDDING, R., AKHURST, R. & KAYA, H., Eds) CSIRO Publications, East Melbourne, Australia, pp. 79–88.

WULFF, A., PETERS, A. & EHLERS, R.-U. (1993) Pathogenicity of the *Steinernema feltiae–Xenorhabdus bovienii* complex to *Tipula oleracea. IOBC/WPRS Bulletin* **17,** 99–102.

XU, J., LOHRKE, S., HURLBERT, I.R. & HURLBERT, R.E. (1989) Transformation of *Xenorhabdus nematophilus. Applied and Environmental Microbiology* **55,** 806–812.

XU, J., OLSON, M.E., KAHN, M.L. & HURLBERT, R.E. (1991) Characterization of Tn5-induced mutants of *Xenorhabdus nematophilus* ATCC 19061. *Applied and Environmental Microbiology* **57,** 1173–1180.

YEH, T. & ALM, S.R. (1992) Effects of entomopathogenic nematode species, rate, soil moisture, and bacteria on control of Japanese beetle (Coleoptera: Scarabaeidae) larvae in the laboratory. *Journal of Economic Entomology* **85,** 2144–2148.

ZHU, Z., LI, G., HE, L. & CHEN, S. (1985). Novel gene transfer into the fertilised eggs of goldfish. *Zeitschrift für Angewandte Ichthyologie* **1,** 31–34.